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Effect of non-operative management (NOM) of splenic rupture *versus* splenectomy on the distribution of peripheral blood lymphocyte populations and cytokine production by T cells

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Summary

Post-traumatic splenectomy is associated with increased postoperative morbidity and mortality and long-term impairment of humoral and cellular immunity. Alternatives to surgery have been developed to minimize or avoid the immediate and/or long-term complications of splenectomy. Herein we investigated the long-term effect of non-operative management (NOM) of the traumatic rupture of the spleen on the distribution of peripheral blood (PB) lymphocyte populations and cytokine production by T cells. PB samples were drawn from six NOM patients, 13 age-matched adults who had undergone splenectomy after trauma (SP patients) and 31 age-matched controls. Cellular phenotypes and the intracellular production of interferon (IFN)-γ, interleukin (IL)-2, IL-4 and IL-10 cytokines in T cells were determined in whole blood ± mitogens by flow cytometry. NOM patients did not show any changes in the absolute numbers of lymphocytes or the distribution of their subsets, compared to the controls. In contrast, SP patients showed a sustained increase in the percentage and/or absolute numbers of lymphocytes, CD8 T cells, activated CD8 T cells, natural killer (NK) T cells, NK cells and γδ T cells, and a reduction in naive CD4 T cells. The constitutive or induced cytokine production by T cells of the NOM group was similar to the control group, whereas SP patients had increased percentages of constitutive IL-2- and IFN-γ-producing CD8 T cells and IFN-γ-producing CD4 T cells. Our findings indicate collectively that the healing process in NOM does not affect the architecture of the spleen to such an extent that it would lead to long-term alterations of the proportions of PB lymphocytes or the T cell cytokine profiles.

Keywords: fluorescence activated cell sorter (FACS), intracellular cytokine staining, phenotype/cell markers, spleen, T cells

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Introduction

Post-traumatic splenectomy is associated with increased postoperative morbidity and mortality and long-term impairment of humoral and cellular immunity [1]. The absence of the phagocytic function of the spleen and the long-term impairment of the humoral response to encapsulated bacteria such as *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* type b are the main causes of the overwhelming post-splenectomy infection syndrome (OPSI) [2]. The capsular polysaccharide antigens of these bacteria elicit an immune response that depends primarily on the function of the splenic marginal zone B cells, but is amplified by factors produced by T cells [2,3]. Because the initiation of the antibody response to polysaccharides depends on the presence of splenic tissue, it is anticipated that its removal will result in a permanent defect. Splenectomized patients, even after immunization, demonstrate suboptimal responses to pneumococcal polysaccharides [4–6]. Although recent reports demonstrate that the currently used 23 polyvalent vaccines mount titres of G and M immunoglobulins in splenectomized individuals that are comparable to those of normal controls, it is not known whether this increase is sufficient to protect splenectomized individuals from OPSI [7–10]. A recent report has shown that OPSI can develop despite adequate titres of IgG antibodies to pneumoccocal antigens, indicating that higher levels of antibodies are required for the elimination of these

Table 1. Clinical characteristics of non-operative management (NOM) and splenectomized patients (SP).

	SP ($n = 13$)	NOM ($n = 6$)
Blood units required	3 (0–15)*	0
ICU stay days	2·3 (0–14)*	0
ISS	22·5 (11–43)*	19·5 (6–35)*
Hospitalization days	11 (5–49)*	12·6 (9–20)*
Age	26 (18–55)*	31 (18–65)*
Years after abdominal trauma	2·5 (1–5)*	2 (1·5–4)*
Male/female	9/4	6/0
Grades II/III/IV	5/4/4	4/1/1

*Median (range). ICU: intensive care unit; ISS: Injury Severity Score.

bacteria in the liver and/or that other aspects of the immune response are affected as well [11]. Few studies have addressed the issue of alterations in T cell immunity in splenectomized individuals. Two published studies reported that splenectomized patients (SP) have impaired primary and memory immune responses to antigens that elicit T cell-dependent responses, indicating that T cell-mediated immunity is also defective in these patients [12,13].

Splenic trauma is an urgent surgical situation in which the haemodynamic stability of the patient is the main criterion for the decision of splenectomy or other surgical spleen-saving technique *versus* non-operative management (NOM). Detailed criteria for assessing the haemodynamic state of these patients have been published in order to help surgeons to follow them up closely and decide surgical management when it is necessary [14]. Considerations of the short- or long-term effect of the immune function of the patients cannot be taken into account in the individual management of patients. However, they helped in the development of alternative approaches to splenectomy in the management of splenic trauma [15].

Earlier studies of patients who underwent partial splenectomy or splenic autotransplantation reported varied effectiveness of primary and recall vaccination with pneumococcal polysaccharides, and OPSI, although rare, remains an issue [6,16,17]. Preclinical studies reported that after using spleen salvage techniques the function of all spleen compartments can be restored to a certain extent, but not completely [18–20], and that the functional capacity of the regenerated splenic tissue depends more on the preservation of the splenic architecture than on the total mass of the implanted tissue [2].

In a preclinical study [21] it was shown that that clearance of *H. influenzae* bacteria and the initial response to pneumococcal polysaccharide vaccines did not differ between rats with splenic trauma managed non-operatively and controls. In this study, the antibody levels decreased significantly 11 days after trauma in NOM rats compared to controls, indicating that immunosuppression associated with trauma *per se* affects the short-term production of antibodies [21]. In a study with children with splenic rupture managed non-operatively, the levels of IgG and IgM antibodies to pneumococcal polysaccharide vaccines did not differ from controls, whereas in splenectomized children the IgM response was defective [22]. Data on T cell-mediated responses in spleen-saving techniques *versus* splenectomized patients are lacking.

The purpose of our study was to investigate the long-term effect of NOM of traumatic rupture of the spleen on the distribution of peripheral blood (PB) lymphocyte populations and cytokine production by T cells, as indicators of normalcy of systemic adaptive immunity.

Materials and methods

Study populations

The clinical parameters of the study subjects are shown in Table 1. They included six adults with a history of rupture of the spleen from blunt abdominal trauma who were treated successfully non-operatively, 13 healthy individuals who had undergone splenectomy after trauma and 31 age-matched healthy adults (17 males, 14 females, median age 37, range: 25–65 years) as controls. For evaluation of the extent of the injury, two scoring systems were used: (i) the grade of splenic rupture was assessed using the Organ Injury Scaling developed by the Organ Injury Scaling Committee of the American Association for the Surgery of Trauma. The scale is graded 1–6 for each organ; 1 is the least severe and 5 the most severe injury the patient can survive. Grade 6 injuries are, by definition, not salvageable [23]. Our study population did not suffer concomitant liver injury that would raise the injury scale and thus depicts accurately the extent of splenic trauma in each of our patients. (ii) The extent of other injuries was assessed using the Injury Severity Score (ISS), an anatomical scoring system that provides an overall score for patients with multiple injuries [24]. Blood smears from all splenectomized individuals were examined prior to the investigation for changes in red cell morphology confirming asplenia, such as the presence of Howell–Jolly bodies, acanthocytes and target cells. To avoid the immediate effect of trauma or other surgery on cellular immunity, the study subjects were investigated at least 1 year after the abdominal trauma (median time 2 years, range 1–5 years). At the time

of the investigation none of the study subjects suffered from any infectious or haematological diseases or was on any medication. The study was approved by the ethical and scientific committee of Patras University Hospital. All subjects included in the study gave their informed consent.

Surface immunophenotyping

The percentages and absolute numbers of the peripheral blood lymphocyte subpopulations were determined by a dual-colour direct immunofluorescence technique applied in whole blood, using the following directly conjugated mouse anti-human monoclonal antibodies (mAbs): CD3-fluorescein isothiocyanate (FITC), phycoerythrin (PE) (clone UCHT1), CD5-FITC (clone BL1a), CD4-FITC (clone 13B8·2), CD8-FITC (clone B9·11), CD20-PE (clone B9E9. HRC20), CD45RA-PE (clone ALB11), CD45RO-PE (clone UCHL1), CD56-PE (clone N901), CD69-PE (clone TP1·55·3), anti-T cell receptor (TCR) γ/δ-PE (clone Immu 510) from Immunotech (Marseille, France), CD25-PE (clone 2A3), anti-human leucocyte antigen D-related (HLA-DR)-PE (clone L243) from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA). After 30 min incubation at 4°C, the red blood cells were lysed and the white blood cells were fixed with the Multi-Q-Prep system (Coulter, Miami, FL, USA). At least 10 000 cells from each sample were analysed by flow cytometry using a Coulter EPICS-XL-MCL cytometer, and the data were processed using XL-2 software.

Intracellular cytokine staining

Intracellular determination of the cytokines interferon (IFN)-γ, interleukin (IL)-2, IL-4 and IL-10 in $CD4^+$ and $CD8^+$ T cells was performed as described [25,26], with modifications: heparinized blood was diluted 1 : 1 with RPMI-1640 medium and cultured ± 25 ng/ml phorbol 12-myristate13-acetate (PMA) and 1 μM ionomycin, in the presence of 10 μg/ml Brefeldin A (BFA) (all from Sigma Chemical Co., St Louis, MO, USA), in 1 ml total volume at 37°C in 5% CO_2 for 5 h. One hundred μl of culture were incubated with the mAb CD8-PC5 (clone B9·11) and the polyclonal antibody CD4-FITC (clone Leu3a-3b; Becton Dickinson Immunocytometry Systems) for 30 min in the dark. After completion of the surface staining, the red blood cells were lysed by incubation with 2 ml of 1× lysing solution for 15 min (PharMLyse; PharMingen, San Diego, CA, USA). The samples were washed with 2 ml phosphate buffered saline (PBS), containing 1% fetal calf serum (FCS) and 0·09% NaN_3 and the cells were permeabilized by incubation with 0·5 ml of Cytofix/Cytoperm buffer (PharMingen) for 20 min at room temperature in the dark, and washed with 2 ml of 1× PermWash Buffer (Pharmingen). The cell pellets were then resuspended and incubated with mAbs to different cytokines for 30 min at room temperature in the dark. The mAbs used were: mouse anti-human IFN-γ-PE (clone 45·15), anti-IL-2-PE (clone N7·48A), anti-IL-4-PE (clone 4D9) from Immunotech, and rat anti-human IL-10-PE (clone JES3–9D7) from PharMingen. The appropriate isotype-matched controls were used to detect non-specific binding and delineate positive and negative populations. The mAb CD69-PE was used as a marker of early activation [27]. Stimulation with PMA/ionomycin resulted in more than 95% intracellular expression of the CD69 antigen in $CD4^+$ and $CD8^+$ T cells. The analysis was performed by a Coulter Epics-XL-MCL flow cytometer. At least 100 000 cells were analysed and the data processed using XL2 software. The analysis was direct for the co-expression of CD8 and CD4 antigens with the various cytokines because the expression of polyclonal CD4 (Leu3a-3b) remains stable after incubation with phorbol esters [28]. The lymphocyte population was defined initially in a forward (FSC) and side scatter (SSC) gate. A second gate (logical) was then created around the $CD4^+$ cells to exclude monocytes from the analysis in a CD4 *versus* SSC dot plot and around the CD8 (bright) cells to exclude natural killers or other cells expressing weakly the CD8 antigen in a CD8 *versus* SSC dot plot. The combination of the two gates for the detection of $CD4^+$ and $CD8^+$ T cells, which accepts only the small lymphocyte population, yields very low background staining for CD69 and cytokines in unstimulated and stimulated samples (< 0·05%). The percentages of cytokine-producing cells were calculated in the total $CD4^+$ or $CD8^+$ T cell populations and not in the total T cell population.

Statistical analysis

Data are expressed as mean ± standard deviation (s.d.). The data were not distributed normally and for this reason non-parametric tests were used to compare values between the three groups. Initial comparisons between the groups were performed using the Kruskal–Wallis test, and when significance arose the Mann–Whitney *U*-test was used for pairwise comparisons. The SPSS version 14, computer program was used for data analysis and *P*-values < 0·05 were considered statistically significant.

Results

Lymphocyte subpopulations

Table 2 shows the detailed immunophenotypic analysis that was performed to determine (i) the absolute numbers and distribution of cell subsets that affect adaptive immunity, i.e. B cells ($CD20^+$), NK T cells ($CD3^+CD56^+$), γδ T cells, T helper cells ($CD3^+CD4^+$), T cytotoxic cells ($CD3^+CD8^+$), naive (CD45RA) and memory (CD45RO) T cells; (ii) the surface expression of HLA-DR, CD25, CD69 activation markers on T cells; and (iii) the proportion of immune cells

Table 2. Distribution of lymphocyte populations in non-operative management (NOM) patients, splenectomized patients (SP) and controls.

Cell type	Controls ($n = 31$)	SP ($n = 13$)	NOM ($n = 6$)	P^* (SP *versus* controls)	P^* (NOM *versus* controls)
Lymphocytes (%)	32 ± 6	37 ± 10	35 ± 6	n.s.	n.s.
cells × 10^9/l	2·07 ± 0·57	2·82 ± 0·67	1·86 ± 0·47	0·004	n.s.
$CD3^+CD4^+$ (%)	47 ± 6	39 ± 7	43 ± 6	0·003	n.s.
cells × 10^9/l	0·94 ± 0·25	1·09 ± 0·30	0·80 ± 0·23	n.s.	n.s.
$CD3^+CD8^+$ (%)	24 ± 7	27 ± 9	22 ± 7	n.s.	n.s.
cells × 10^9/l	0·49 ± 0·19	0·81 ± 0·35	0·43 ± 0·22	0·010	n.s.
$CD4^+CD45RA^+$ (%)	20 ± 7	14 ± 5	23 ± 12	0·010	n.s.
cells × 10^9/l	0·43 ± 0·18	0·4 ± 0·18	0·42 ± 0·22	n.s.	n.s.
$CD4^+CD45RO^+$ (%)	29 ± 7	27 ± 8	25 ± 9	n.s.	n.s.
cells × 10^9/l	0·61 ± 0·23	0·75 ± 0·25	0·47 ± 0·19	n.s.	n.s.
$CD8^+CD45RA^+$ (%)	18 ± 5	21 ± 4	20 ± 6	n.s.	n.s.
cells × 10^9/l	0·39 ± 0·14	0·59 ± 0·16	0·37 ± 0·13	0·001	n.s.
$CD8^+CD45RO^+$ (%)	10 ± 4	12 ± 4	9 ± 4	n.s.	n.s.
cells × 10^9/l	0·22 ± 0·15	0·35 ± 0·18	0·17 ± 0·09	0·008	n.s.
$CD3^+CD56^+$ (%)	2·9 ± 3·2	4·8 ± 3·2	2·2 ± 1·6	0·012	n.s.
cells × 10^9/l	0·07 ± 0·07	0·13 ± 0·09	0·04 ± 0·03	0·002	n.s.
$CD3^-CD56^+$ (%)	9 ± 3	13 ± 6	11 ± 5	n.s.	n.s.
cells × 10^9/l	0·2 ± 0·1	0·36 ± 0·15	0·2 ± 0·09	0·0001	n.s.
$CD4^+CD69^+$ (%)	0·2 ± 0·1	0·3 ± 0·3	0·1 ± 0·1	n.s.	n.s.
cells × 10^9/l	0·004 ± 0·004	0·008 ± 0·012	0·003 ± 0·002	n.s.	n.s.
$CD8^+CD69^+$ (%)	0·3 ± 0·2	0·6 ± 0·6	0·4 ± 0·1	n.s.	n.s.
cells × 10^9/l	0·006 ± 0·003	0·018 ± 0·025	0·007 ± 0·003	0·007	n.s.
$CD4^+CD25^+$ (%)	1·1 ± 0·7	1·5 ± 0·9	1·1 ± 0·7	n.s.	n.s.
cells × 10^9/l	0·023 ± 0·014	0·046 ± 0·030	0·018 ± 0·011	0·014	n.s.
$CD4^+$HLA-DR^+ (%)	1·1 ± 0·5	1 ± 0·4	0·7 ± 0·4	n.s.	n.s.
cells × 10^9/l	0·022 ± 0·009	0·030 ± 0·014	0·019 ± 0·004	n.s.	n.s.
$CD8^+$HLA-DR^+ (%)	0·5 ± 0·2	0·5 ± 0·2	0·4 ± 0·2	n.s.	n.s.
cells × 10^9/l	0·011 ± 0·007	0·016 ± 0·005	0·007 ± 0·004	0·019	n.s.
$CD20^+$ (%)	13 ± 3	11 ± 4	12 ± 2	n.s.	n.s.
cells × 10^9/l	0·26 ± 0·10	0·30 ± 0·14	0·23 ± 0·05	n.s.	n.s.
$CD20^+CD5^+$ (%)	0·6 ± 0·4	0·7 ± 0·6	0·9 ± 0·3	n.s.	n.s.
cells × 10^9/l	0·013 ± 0·008	0·021 ± 0·018	0·017 ± 0·004	n.s.	n.s.
TCR-γδ	5 ± 2	6 ± 1	5 ± 3	n.s.	n.s.
cells × 10^9/l	0·10 ± 0·06	0·18 ± 0·08	0·09 ± 0·05	0·024	n.s.

Lymphocyte phenotypes were determined in whole blood by dual-colour immunofluorescence flow cytometry. Data are expressed as mean ± standard deviation. n.s.: statistically non-significant difference. *The Mann–Whitney *U*-test was used for comparison of controls with SP and controls with NOM.

that reside primarily in the spleen, i.e. $CD20^+CD5^+$B cells and NK ($CD3\text{-}CD56^+$) cells.

In NOM patients, the number and percentage of lymphocytes and the distribution of lymphocyte subsets (as absolute numbers and percentages) were within control range (Table 2). Splenectomized patients (SP) had higher absolute number of their total lymphocyte population, compared to controls and NOM (differences statistically significant, Table 2). This lymphocytosis was due mainly to an increase in the absolute numbers of $CD8^+$ and NK cells and, to a lesser extent, of NK T and γδ T cells. The observed increase in the absolute number of $CD8^+$ T cells was due to an increase in both $CD8^+CD45RO^+$ (memory) and $CD8^+CD45RA^+$ (naive) subsets. In the same patients, the changes in the absolute numbers of their various subsets resulted in a redistribution of PB lymphocytes with decreased percentages of $CD4^+$ T cells and naive $CD4^+CD45RA^+$ T cells and an increased percentage of NK T cells. Finally, SP patients had increased absolute numbers of activated $CD8^+$ T cells compared to controls and NOM, as indicated by the increased surface expression of CD25, HLA-DR and CD69 antigens (Table 2).

In vivo intracellular production of T helper 1 (Th1) and Th2 cytokines

The study of the intracellular cytokine expression of unstimulated T cells showed that the constitutive production of Th1 and Th2 cytokines by $CD4^+$ and $CD8^+$ T cells of NOM patients was within control range. In contrast, post-traumatic splenectomy resulted in constitutive production of type 1 cytokines, demonstrated by an increase in the

Table 3. Percentages of T helper 1 (Th1) [interferon (IFN)-γ, interleukin (IL-2)]- and Th2 (IL-4, IL-10)-producing unstimulated $CD4^+$ and $CD8^+$ T cells from SP ($n = 13$), non-operative management (NOM) patients ($n = 6$) and controls ($n = 17$).

Cytokine	Group	$CD4^+$ T cells (%)	*P*-value*	$CD8^+$ T cells (%)	*P*-value*
IFN-γ	Controls	0·08 ± 0·04		0·08 ± 0·04	
	SP	0·14 ± 0·06	0·020	0·24 ± 0·12	0·001
	NOM	0·12 ± 0·04	n.s.	0·09 ± 0·04	n.s.
IL-2	Controls	0·16 ± 0·08		0·12 ± 0·07	
	SP	0·24 ± 0·14	n.s.	0·28 ± 0·17	0·011
	NOM	0·21 ± 0·12	n.s.	0·10 ± 0·08	n.s.
IL-4	Controls	0·13 ± 0·08		0·10 ± 0·05	
	SP	0·15 ± 0·07	n.s.	0·14 ± 0·08	n.s.
	NOM	0·15 ± 0·09	n.s.	0·09 ± 0·02	n.s.
IL-10	Controls	0·25 ± 0·11		0·31 ± 0·14	
	SP	0·39 ± 0·34	n.s.	0·38 ± 0·29	n.s.
	NOM	0·42 ± 0·24	n.s.	0·36 ± 0·27	n.s.

*The Mann–Whitney *U*-test was used to compare controls with SP and controls with NOM. Whole blood was cultured for 5 h in plain culture medium + Brefeldin A. The cytokine-producing cells were calculated as percentages of the $CD4^+$ or $CD8^+$ T cell populations. The data are expressed as mean ± standard deviation. n.s.: statistically non-significant difference.

percentage of IL-2 and IFN-γ-producing $CD8^+$ cells and IFN-γ-producing $CD4^+$ cells (Table 3 and Fig. 1). These results, taken together with the observation that there is increased surface expression of activated markers, indicate that there is *in vivo* priming of T cells in the SP group.

Intracellular production of Th1 and Th2 cytokines following mitogenic stimulation *in vitro*

Following stimulation with PMA/ionomycin, a strong Th1 profile emerged in all study groups (Table 4 and Fig. 1). IL-2

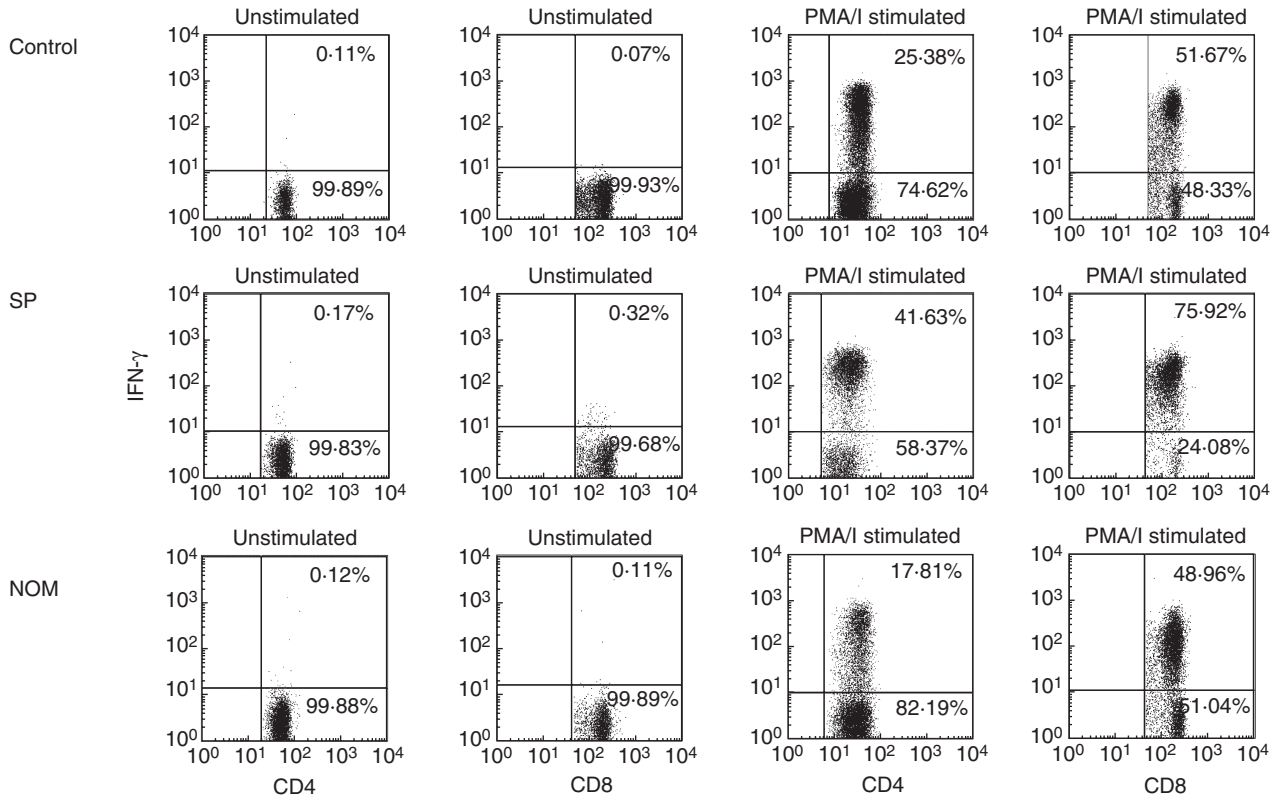

Fig. 1. A representative fluorescence activated cell sorter experiment showing the percentage of interferon-γ-producing $CD4^+$ and $CD8^+$ T cells in a non-operative management patient (bottom), a splenectomized individual (middle) and a control (top). Unstimulated samples, whole blood cultured for 5 h in plain medium + Brefeldin A; phorbol 12-myristate13-acetate (PMA)/I-stimulated samples, whole blood cultured for 5 h with PMA/I in the presence of Brefeldin A.

Table 4. Percentages of T helper 1 (Th1) [interferon (IFN)-γ, interleukin (IL)-2]- and Th2 (IL-4, IL-10)-producing $CD4^+$ and $CD8^+$ T cells from splenectomized patients (SP) ($n = 13$), non-operative management (NOM) patients ($n = 6$) and controls ($n = 17$), following mitogenic stimulation.

Cytokine	Group	$CD4^+$ T cells (%)	*P*-value*	$CD8^+$ T cells (%)	*P*-value*
IFN-γ	Controls	21·62 ± 6·81		49·34 ± 13·54	
	SP	30·29 ± 11·11	0·068	63·27 ± 18·97	0·059
	NOM	20·24 ± 2·23	n.s	43·2 ± 12·67	n.s.
IL-2	Controls	65·38 ± 6·07		20·97 ± 6·77	
	SP	58·82 ± 16·36	n.s.	17·23 ± 8·07	n.s.
	NOM	67·56 ± 4·50	n.s.	23·64 ± 6·61	n.s.
IL-4	Controls	0·28 ± 0·17		0·15 ± 0·11	
	SP	0·27 ± 0·13	n.s.	0·14 ± 0·07	n.s.
	NOM	0·34 ± 0·11	n.s.	0·11 ± 0·08	n.s.
IL-10	Controls	0·51 ± 0·17		0·37 ± 0·16	
	SP	0·80 ± 0·41	n.s.	0·31 ± 0·07	n.s.
	NOM	0·47 ± 0·24	n.s.	0·29 ± 0·05	n.s.

Whole blood samples were cultured with phorbol 12-myristate13-acetate/I for 5 h in the presence of Brefeldin A. The cytokine-producing cells were calculated as percentage of the $CD4^+$ or $CD8^+$ T cell populations. The data are expressed as mean ± standard deviation. n.s.: statistically non-significant difference. *The Mann–Whitney *U*-test was used to compare controls with SP and controls with NOM.

production predominated in $CD4^+$ T cells and IFN-γ in $CD8^+$ T cells. The intracellular cytokine pattern did not differ between NOM patients and controls (Table 4). In contrast, the percentage of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells were higher in SP patients compared to the NOM and control groups, although this increase did not reach statistical significance (Table 4 and Fig. 1). The percentages of Th2 cytokine-producing T cells remained low after mitogenic stimulation in all groups and no significant differences were observed among them (Table 4).

Discussion

In the present study parameters of T cell immunity of patients with splenic trauma managed non-operatively were compared to those of splenectomized patients at least 1 year after trauma. In NOM patients the absolute numbers and distribution of PB lymphocyte subsets were within control range. In contrast, post-traumatic splenectomy resulted in a significant increase of the absolute numbers and alteration of the proportion of various lymphocyte populations in PB of otherwise healthy individuals. These changes underlie the importance of spleen as a cytotoxic T cell reservoir and its role in the regulation of the trafficking of PB lymphocytes [29]. In experimental animals, the changes in the distribution of T cells that occur after splenectomy are temporary [30], whereas in humans they last for a long time and are perhaps irreversible [26]. Similarly to us, other investigators have reported a long-term reduction in the percentage of $CD4^+$ T cells and/or a reduction in the CD4/CD8 ratio and an increase of NK cell numbers after splenectomy [13,31–35].

The effect of the changes in the absolute numbers and distribution of lymphocyte subsets on the immune response of SP patients is not clear. A previous study investigating a similar age group of splenectomized individuals found also a long-term reduction of the percentage of naive $CD4^+$ T cells, and linked this reduction to primary T cell defects resulting to a weak response to hepatitis A vaccination [12].

In splenectomized, but otherwise healthy, individuals the observed T cell activation and cytokine production indicate *in vivo* priming of T cells, especially $CD8^+$ T cells. *In vivo* priming of T cells producing type 1 cytokines may result in increased production of IFN-γ and activation of cells of the innate immunity, in agreement with previous studies showing increased cytotoxic activity of NK cells and activation of monocytes in splenectomized individuals [31,36,37].

Persistent increase in absolute numbers and activation of cytotoxic T and NK cells may compensate in part for the lack of phagocytic activity in the spleen but may also participate in the development of post-splenectomy complications, such as chronic pulmonary hypertension, due to an increased production of proinflammatory cytokines [38].

Previous studies showed that peripheral blood mononuclear cells (PBMC) from SP patients have an increased spontaneous production of immunoglobulins but a reduced production thereof after stimulation of the cells with the T cell-specific mitogen pokeweed [39,40]. This defective production of Ig in PBMC + pokeweed cultures could be restored when B cells derived from SP patients were co-cultured with T cells derived from controls [33]. It can be suggested, therefore, that the observed T cell impairment in SP patients is due to *in vivo* activation of T cells that, in turn, leads to an increased spontaneous production of immunoglobulin by PBMC. This continuous *in vivo* priming may lead to a state of 'exhaustion' of memory T cells [41], a fact that may explain data showing *in vivo* defective responses to recall antigens after vaccination post-splenectomy [13].

Published studies on the immune responses in NOM patients are scarce. Our findings indicate collectively that the healing process in NOM does not affect the architecture of the organ to such an extent that it would lead to long-term

alterations of the proportions of PB lymphocytes or the T cell cytokine profiles. Therefore, there is a strong possibility that the adaptive immune response remains quite intact in NOM patients.

Karp *et al*. have shown that, although the initial response to pneumococcal polysaccharide vaccines did not differ between rats with splenic trauma managed non-operatively and controls, 11 days after trauma there was a significant decrease in antibody levels in NOM rats [21]. It is well documented that trauma results in immunosuppression [42], but our data collected at least a year after trauma show that in humans this result is short term and does not affect the long-term immunity.

There are concerns regarding the immediate and long-term immunosuppressive effects of red blood cell (RBC) transfusions in surgical patients [43]. This is an issue that our study cannot address, because none of the participating NOM patients received any RBC transfusion (Table 1).

In conclusion, in patients with splenic rupture who underwent splenectomy, there is a long-term change in T cell immunity whereas in NOM patients no long-term changes were observed in the parameters studied. These findings do not exclude a temporal disturbance of cellular immunity in NOM patients during the first months after the trauma.

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